Chlamydia pneumoniae Induces Chemokine Expression by Platelets in Patients with Atherosclerosis

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Key Words

Platelets · Atherosclerosis · CCL3 · CCL5 · CCL7 · CXCL8

Abstract

Objective: In this study, the role of *Chlamydia pneumoniae* in triggering platelets to induce the inflammatory potential chemokines CCL3, CCL5, CCL7 and CXCL8 in atherosclerotic patients was investigated. **Subjects and Methods:** Venous blood from control subjects (n = 35) and atherosclerotic patients (n = 35) was collected in tubes with and without EDTA. Platelets from controls and patients were separated from whole blood and then stimulated with lipopolysaccharide (LPS), live *C. pneumoniae* and heat-treated *C. pneumoniae*. The ability of *C. pneumoniae* and its LPS to stimulate platelets and expression of CCL3, CCL5, CCL7 and CXCL8 was assessed with immunofluorescence. Immunosorbent assays were used to detect anti-*C. pneumoniae* antibodies in sera from patients and healthy subjects. **Results:** Nonstimulated platelets from patients showed significant expression of CCL3, CCL5, CCL7 and CXCL8 compared to controls (p < 0.0001). Stimulation of platelets from patients with live and heat-treated *C. pneumoniae* and its LPS demonstrated significant induction of chemokines compared to similarly stimulated platelets from controls (p < 0.01). After stimulation with heat-treated *C. pneumoniae* chemokine expression in platelets

from controls was significantly lower than after stimulation with live *C. pneumoniae* (p < 0.01), which was not the case when platelets from patients were stimulated. Increased levels of anti-*C. pneumoniae* antibodies were detected in sera from patients compared to healthy subjects, suggesting prior *C. pneumoniae* exposure. **Conclusion:** Our data demonstrated an interactive link between *C. pneumoniae* and platelets in atherosclerotic patients, leading to induction of potential chemokines and possibly disease development.

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Introduction

Atherosclerosis is a progressive disease in which inflammatory cells, activated smooth muscle cells, lipids, and extracellular matrix accumulate in the arterial wall, resulting in growth of plaques. Atherosclerosis is now viewed as an inflammatory disease of the vascular system, with macrophages, lymphocytes, and platelets being important sources of cytokines and growth factors that control the migration, proliferation, and activation of smooth muscle cells and monocytes leading to intimal hyperplasia [1].

Chlamydia pneumoniae is an obligate intracellular bacterium responsible for a number of upper and lower

respiratory tract diseases in humans, and approximately 50% of adults worldwide have antibody evidence of previous infection by this bacterium [2]. An association between *C. pneumoniae* infections and atherosclerosis has been demonstrated in a number of epidemiological, serological, immunohistochemical, and molecular biological investigations [3, 4]. In vitro studies have revealed that *C. pneumoniae* is able to infect and replicate in the major cell types found in atherosclerotic lesions, such as macrophages, endothelial cells, and smooth muscle cells [5, 6].

Platelets are un-nucleated cellular fragments that circulate in the blood. In addition to their well-recognized role in hemostasis and acute thrombus formation, platelets are also thought to have proinflammatory and growth-regulatory properties that contribute to progression of atherosclerosis [7, 8].

C. pneumoniae binds to platelets and triggers aggregation, secretion, and surface expression of P-selectin, a facilitator of atherosclerosis [9]. Also, *C. pneumoniae* is able to stimulate platelets leading to reactive oxygen species expression, which may play a crucial role in the development of atherosclerosis [10].

Chemokines are a superfamily of chemotactic cytokines [11]. They activate and direct the migration of leukocytes by binding to specific G protein-coupled 7-transmembrane cell surface receptors [12]. Several studies suggest that chemokines play a pathogenic role in atherosclerosis by activating and recruiting inflammatory cells to atherosclerotic lesions [13, 14].

In this work, we evaluated the interaction between *C. pneumoniae*, platelets and potential chemokines to study a possible pathogenetic mechanism that might be involved in the development of atherosclerosis.

Subjects and Methods

Patients and Controls

Venous blood samples were collected from atherosclerotic patients who underwent clinically indicated assessment for coronary artery or peripheral vascular disease in Bahrain in 2007–2008 (n = 35) and from healthy blood donors (controls; n = 35) in tubes with and without EDTA. All subjects were asked to fill in a consent form and sign it, indicating their acceptance to participate in the study. The characteristics of both patients and controls are listed in table 1.

Cells and Bacteria

C. pneumoniae was isolated from the patients and identified using polymerase chain reaction with specific primers and DNA sequencing as previously described [9]. Human platelets were isolated from whole blood according to the Springer Lab Protocol

Table 1. Clinical characteristics of patients and healthy controls

	Controls (n = 35)	Patients (n = 35)	p
Men/women	20/15	18/17	
Age, years	43 ± 5	55 ± 7	<0.25
BMI, kg/m ²	24.3 ± 1.0	28.9 ± 0.8	<0.15
Leukocytes/μl	5,850 ± 625	6,150 ± 350	<0.10
PLT, 10 ⁴ /μl	24.5 ± 4.8	25.5 ± 6.2	<1.05
T-CHOL, mmol/l	14.13 ± 0.20	4.68 ± 0.33	<0.09
HDL-CHOL, mmol/l	1.25 ± 0.06	0.90 ± 0.06	<0.05
LDL-CHOL, mmol/l	2.13 ± 0.10	2.93 ± 0.21	<0.005
LDL/HDL ratio	1.70	3.26	<0.0001
TG, mmol/l	3.77 ± 0.91	4.29 ± 1.17	<0.35

BMI = Body mass index; PLT = platelet count; T-CHOL = total cholesterol; HDL-CHOL = high-density lipoprotein cholesterol; LDL-CHOL = low-density lipoprotein cholesterol; TG = triglycerides.

(Springer Lab separation of platelets from whole blood: Azucena Salas, Springer Lab, The CBR Institute for Biomedical Research, Inc., Boston, Mass., USA). The multiplicity of infection was approximately 0.1. To demonstrate the purity of the isolation procedure staining for the specific-platelet marker CD61 was performed as described below using the mouse anti-human CD61 (AbD Serotec, Kidlington, UK).

Stimulation of Platelets with Live, Heat-Treated *C. pneumoniae* and Lipopolysaccharide

One adhesive slide (BioRad, Munich, Germany) was needed per patient or control subject to detect each of the four chemokines (CCL3, CCL5, CCL7 or CXCL8). A 300-μl sample of platelet suspension containing 10⁴ cells of each patient or control was transferred to each well. Platelet suspension was stimulated with a final concentration of 25 pg/ml lipopolysaccharide (LPS, kindly donated by Dr. Meytham Majeed, Department of Microbiology, University of Linköping, Sweden), 20 μl of live and 20 μl of heat-treated *C. pneumoniae* (at 70°C for 30 min). Some wells of each slide were left without stimulation (negative control). All slides were incubated overnight at room temperature.

Immunofluorescence

The cellular instead of serum chemokines were measured because the serum level may not be accurate because chemokines have a short half-life and bind with high affinity to nearby receptors.

The supernatant was aspirated carefully, 50 μl of ice-cold 4% paraformaldehyde was added to fix the cells and the cells were incubated at 4°C for 20 min. The cells were washed with phosphate-buffered saline (1× PBS) to remove free ice-cold 4% paraformaldehyde, and 25 μl of 2% fetal bovine serum in 1× PBS was added to block unbound surface areas of all slides. The cells were incubated at room temperature for 15 min and then washed twice in 1× PBS-0.1% saponin. Five micrograms (15 μl/well) of 0.1 mg/ml mouse anti-human CCL5 (AbD Serotec, Kidlington, UK,

MCA 1559) diluted 1:100 in 1× PBS-0.1% saponin supplemented with 5% normal donkey serum (C06SB2) was added to all wells (8 wells) of the first slide; 5 µg (15 µl/well) of 0.5 mg/ml mouse anti-human CCL3 (AbD Serotec, Kidlington, UK, MCA 1819) diluted 1:100 in 1× PBS-0.1% saponin supplemented with 5% normal donkey serum (C06SB2) was added to all wells (8 wells) of the second slide; 5 µg (15 µl/well) of 0.5 mg/ml mouse anti-human CCL7 (AbD Serotec, Kidlington, UK, MCA 1818) diluted 1:100 in 1× PBS-0.1% saponin supplemented with 5% normal donkey serum (C06SB2) was added to all wells (8 wells) of the third slide, and 5 µg (15 µl/well) of 0.5 mg/ml mouse anti-human CXCL8 (AbD Serotec, Kidlington, UK, MCA 1109) diluted 1:100 in 1× PBS-0.1% saponin supplemented with 5% normal donkey serum (C06SB2) was added to all wells (8 wells) of the fourth slide. An isotype-matched control antibody was used for the chemokine CCL5 as a control. The cells were washed twice with 1× PBS-0.1% saponin, and the cells were incubated at room temperature for 1 h in the dark with 15 µl of 2 mg/ml Alexa Fluor donkey anti-mouse IgG (H + L) (500-M73) dilutes 1:300 in 1× PBS-0.1% saponin supplemented with 5% normal donkey serum. All wells of the four slides were washed twice in 1× PBS-0.1% saponin and air-dried. The slides were mounted using anti-fading mounting medium. The immunopositive platelets were examined under a fluorescent microscope (Nikon, Evlipss-E600) and photographed by Nikon digital camera. Chemokine expression in platelets was counted manually using ×40 objective. Chemokine expression by platelets was assessed by examining at least 10⁴ platelets.

Enzyme Immunosorbent Assay

A kit was used to detect anti-*C. pneumoniae* antibody (*C. pneumoniae* IgG kit, product No. 6111300, Ani Labsystems Ltd. Oy, Vantaa, Finland). It was based on an indirect solid-phase enzyme immunoassay with horseradish peroxidase as a marker enzyme. The assay was done according to manufacturer's instructions and the reaction was measured at 450 nm.

Analysis

To examine in vivo the constitutive expression of potential proinflammatory chemokines in platelets from patients with atherosclerosis, we looked for spontaneous chemokine induction on these cells compared to those of healthy subjects. The examined chemokines were selected in view of their prospective roles in inflammation and to further evaluate the interaction between *C. pneumoniae*, platelets and these chosen chemokines to study a possible pathogenetic mechanism that might be involved in the development of atherosclerosis.

Statistical Analysis

Student's t test was used to test the level of significance. If the p value was ≤ 0.05, the difference was accepted as statistically significant. Sigmasat package was used for the statistical analysis.

Results

The characteristics of atherosclerotic patients and healthy subjects including gender, age, body mass index, leukocytes, platelet counts, total cholesterol, high-density

lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides are shown in table 1.

Nonstimulated platelets from patients showed significant expression of CCL3, CCL5, CCL7 and CXCL8 compared to controls ($p < 0.0001$; fig. 1, 2a, b).

The stimulation of platelets from patients with live, heat-treated *C. pneumoniae* and LPS of *C. pneumoniae* demonstrated significant induction of chemokines compared to similar stimulation of platelets from controls ($p < 0.01$; fig. 1). However, the stimulation of platelets from healthy subjects revealed significant expression of all examined chemokines ($p < 0.0001$) compared to non-stimulated platelets, which at the same time was significantly lower than chemokine expression in stimulated platelets from patients ($p < 0.0001$). Stimulation with live *C. pneumoniae* was significantly higher than stimulation with heat-treated *C. pneumoniae* for chemokine expression in platelets from controls ($p < 0.01$). With live *C. pneumoniae*, 1,200 ± 95 CCL3, 1,250 ± 110 CCL5, 1,450 ± 50 CCL7, and 1,100 ± 65 CXCL8 immunopositive platelets per 10⁴ cells were found compared to 850 ± 100 CCL3, 900 ± 120 CCL5, 1,200 ± 120 CCL7, and 900 ± 85 CXCL8 immunopositive platelets per 10⁴ cells when heat-treated *C. pneumoniae* was used. However, this was not the case when platelets from patients were stimulated, where lower levels were detected without reaching statistical significance (fig. 1).

Staining for the specific platelet marker (CD61) to demonstrate the purity of the isolation procedure is shown in figure 2c. Also, an isotype-matched control antibody was used for the chemokine CCL5 as a control and no staining was detected (fig. 2d).

To detect any previous exposure of atherosclerotic patients to *C. pneumoniae* infection, we looked for anti-*C. pneumoniae* antibody titers in sera in comparison to healthy subjects. Significantly increased levels of anti-*C. pneumoniae* antibodies were detected in sera from patients compared to healthy controls ($p < 0.0001$), suggesting prior *C. pneumoniae* infection in atherosclerotic patients (fig. 3).

Discussion

Our current study confirmed the notion that proinflammatory chemokines play a key role in the inflammatory process of atherosclerosis and that *C. pneumoniae* is a triggering agent. A growing body of evidence suggests that *C. pneumoniae* has a role in the development of atherosclerosis [3, 15, 16]. However, it is uncer-

Fig. 1. Expression of CCL3 (a), CCL5 (b), CCL7 (c) and CXCL8 (d) by platelets stimulated with LPS, live *C. pneumoniae* and heat-treated *C. pneumoniae*. The bars represent means $\pm$ SD from a procedure run in triplicate. These values were compared to those of healthy subjects and analyzed by Student's t test (* $p < 0.1$, ** $p < 0.001$, *** $p < 0.0001$).

tain whether a *C. pneumoniae* infection is a triggering event of atherosclerosis or a secondary infectious complication of an already formed atherosclerotic plaque. Several studies [13, 14] have investigated *C. pneumoniae* interaction with different cell types involved in the atherosclerotic process, for example, monocytes/macrophages, smooth muscle cells, and endothelial cells, but the interaction with platelets, which might lead to chemokine induction and promotion of atherosclerosis, was not explored.

C. pneumoniae infection is very common among the human population and it occurs early in life, causing the bacteria to persist for long periods in tissues [15]. Thus, there are probably several opportunities for *C. pneumo-*

niae-platelet interaction, which may stimulate both the early proliferative phases of atherosclerosis and late thrombotic vascular occlusion. Platelets are a significant cell type involved in this process. Besides their role in hemostasis, platelets act as immune cells that potentiate vascular inflammation. They may adhere to intact endothelial cells and promote local vascular inflammation by recruiting leukocytes via direct interaction or by secreting inflammatory mediators such as chemokines, leading to vascular injury or dysfunction, thereby contributing to neointimal hyperplasia or atherosclerosis [14].

C. pneumoniae binds to platelets and triggers P-selectin, a facilitator of atherosclerosis [9]. Also, *C. pneumo-*

Fig. 2. Fluorescent photographs showing chemokine expression. Dark field fluorescent microscopy. $\times 40$. **a** Example of CCL5 in platelets from an atherosclerotic patient without stimulation. **b** Example of CCL5 in nonstimulated platelets from a healthy subject (negative control). **c** Staining for specific platelet markers (CD61) to demonstrate the purity of the isolation procedure. **d** CCL5 isotype-matched control antibody staining.

niae interaction with platelets has been shown to induce aggregation, reactive oxygen species production and an oxidative effect on low-density lipoprotein and to have a crucial role in the development of atherosclerotic cardiovascular disease [10]. Previous work [17] showed that platelet activation leads to the release of α -granule chemokines, including CCL3, CCL5, CCL7, CCL17, CXCL1, CXCL5 and CXCL8, which attract leukocytes and further activate other platelets providing evidence of a direct association between hemostasis, infection, and inflammation and the development of atherosclerosis. Our current work has, furthermore, identified *C. pneumoniae* as a critical infectious agent in this process. We noted that *C. pneumoniae* infection can induce chemokine expression in control platelets, and to a lesser degree in patient platelets that already showed spontaneous chemokine expression. This is most probably due to

Fig. 3. Antibodies as measured by enzyme immunoassay units (EIU) for sera from patients and healthy subjects. Bars represent means $\pm$ SD. The values were compared to those of healthy subjects and analyzed by Student's *t* test (***) $p < 0.0001$.

prior infection with *C. pneumoniae* that elicited a high level of chemokines in patient platelets and further infection that could additionally increase the chemokine level.

The serological response to *C. pneumoniae* infection is characterized by two different patterns. Primary infection with *C. pneumoniae* produces an IgM titer rise followed by IgG increase, whereas reinfection with *C. pneumoniae* produces a rise in IgG and IgA titer without IgM increase. *C. pneumoniae*-specific IgG antibodies were our target in atherosclerotic patients [18]. The results of anti-*C. pneumoniae* antibody titers in patient sera showed high IgG antibody titers in comparison with healthy controls, suggesting prior *C. pneumoniae* exposure.

Conclusion

Our findings demonstrate the induction of potential chemokines, namely CCL3, CCL5, CCL7 and CXCL8, by platelets in response to *C. pneumoniae*, which might be considered as an interactive process in the development of atherosclerosis and thrombotic vascular occlusion. Further studies on the mechanisms of communication and signaling pathways are essential for any future therapeutic intervention plans.

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